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Production of bacterial cellulose by *Gluconacetobacter sacchari* using dry olive mill residue

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ABSTRACT

Bacterial Cellulose (BC), produced by many bacteria specially those belonging to the *Gluconacetobacter* genus, is a very peculiar cellulose form that bears unique mechanical and structural properties that can be exploited in numerous applications. However, the production costs of BC are very high because of the use of quite expensive culture media. In this sense, the purpose of this work was to evaluate the possibility of use residues from the olive oil production industry as nutrient and carbon source for the production of BC by *Gluconacetobacter sacchari*. The dry olive mill residue (DOR) was submitted to water extraction at 40 and 100 °C (DOR40 and DOR100) and to hydrolysis with H_2SO_4 1M (DOR100H) in order to obtain sugar rich aqueous extracts to be used for BC production. The BC production obtained without addiction of any type of nutrients was 0.81 g L^{-1} for DOR40 and 0.85 g L^{-1} for DOR100 after 96 h incubation, which corresponded respectively to 32 and 34% of the production achieved with conventional HS culture medium (around 2.5 g L^{-1}). In order to enhance the production of BC, the residues were supplemented with nitrogen (N) and phosphate (P) sources to overcome possible nutritional limitations. It was verified an increase on the BC production between 21.5% (N8 P4,5) and 43.2% (N1 P8) when compared with no supplementation. These are promising results to overcome high BC production costs.

1. Introduction

Cellulose is one of the most abundant natural polymers on Earth [1], most of which produced by plants (VC). However cellulose can also be produced by microorganisms such as algae and bacteria. Several bacteria belonging to different genera like *Gluconacetobacter*, *Sarcina* and *Agrobacterium* are able to produce a unique form of cellulose designated as bacterial cellulose, or biocellulose (BC) [2]. Recently, *Gluconacetobacter sacchari* was identified as a very efficient BC producer [3]. Since these bacteria are aerobic, under oxygen limiting conditions, like static medium, the production of BC takes place in the liquid–gas interface in the form of a highly swollen membrane.

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Bacterial cellulose presents high purity, since it is not associated with other components such lignin and hemicelluloses as in VC [4], and due to its 3D nanofibrillar network [5,6] shows high water absorption capacity (the original membrane possesses ~99% water) and high tensile strength.

These singular properties together with other interesting features, namely biocompatibility [5,7], biodegradability and renewable character result in a wide range of BC applications, such as in the biomedical area, e.g. in wound dressing for recovering burned skin [5,7], as membranes for dermal drug delivery [8], artificial blood vessels for microsurgery [4], polymeric scaffolds for bone and cartilage repair [9,10] and in several technological fields such as membranes for audio devices [6], electronic paper [11], as reinforcement material in transparent/translucent nanocomposites [12–14] and paper coating [15], among others.

Despite its enormous potential, and with exception of biomedical applications, the high BC production costs, associated with the use of expensive culture media, is the main factor hampering the large scale development of BC applications, especially for lower added value products such as in paper coating industry and in some composite materials.

This drawback can be overcome by two distinct approaches: Notably, the genetic modification of microorganisms to improve microorganism productivity or, through the use of inexpensive industrial by-products and residues to replace not only glucose as carbon source, but also other nutritional components of the culture media, reducing the production costs. The first approach has been tested successfully [16], however the use at a commercial scale of products from genetically modified organisms, particularly in biomedical and cosmetic Applications, is more complex than those derived from natural and non-pathogenic microorganisms.

Concerning the second approach, several industrial wastes have already been successfully investigated for the production of BC, as for example Konjac powder [17], beet molasses [18], sugar-cane molasses and corn steep liquor [19] and several fruit juices, including orange, pineapple, apple, Japanese pear and grape [20], as well as coconut water [21]. More recently, we have tested several residues from agro-forestry industries, namely grape skins aqueous extract, cheese whey, crude glycerol and sulfite pulping liquor, as low cost sources of carbon and nutrients (with or without supplementation), for the production of BC by *G. sacchari* [22]. The potential of using this type of wastes as substrates for the production of this valuable biopolymer simultaneously with the valorization of the residues themselves was demonstrated.

Olive oil production is an important economic activity in Portugal and elsewhere in the Mediterranean basin [23,24]. Industrial olive oil extraction generates every year, between October and January, around 40 kt of a high water content solid residue, the so called two-phase olive pomace (OP), composed of skin, pulp and stone pieces of olive fruit [25] that can cause serious environmental problems if not adequately treated. OP is usually dried at high temperatures (400–800 °C) and then submitted to hexane extraction in order to obtain the OP oil, which accounts for ~9.2% of the dry original OP [26], generating another by-product named dry olive mill residue (DOR) [27]. The final residue, DOR, which accounts for ~35% of the mass of the dry original OP [26], is normally burned for cogeneration of electric power. Other less important applications of these residues include their application as organic fertilizers [28] or animal feeding supplements [29].

However, in addition to oil extraction and energetic conversion these residues could be substantially valorized if valuable bioactive compounds [30,31] or nutrients [28] are extracted from DOR before final burning.

Following our interest on finding agro-forestry residues that could be used as carbon and nutrient sources for the production of BC, in the present paper we have studied the potential of DOR, after mild acid hydrolysis, in order to obtain a substrate rich in monomeric sugars as carbon and nutrient source for the production of BC.

2. Materials and methods

2.1. Reagents

Glucose (96% purity), xylose (98% purity), arabinose (98% purity), fructose (99% purity), galactose (97% purity), Na_2HPO_4 (98% purity), KH_2PO_4 (99% purity) and $(NH_4)_2SO_4$ (99% purity) were purchased from Sigma Chemicals. Yeast extract and bacteriological peptone were purchased from Himedia, and citric acid was obtained from Acros Organics. All other chemicals were of analytical grade.

2.2. Olive oil industrial residues

The olive mill industrial residue (DOR), used in the present study, was provided by Mariano Lopes & Filhos, Lda (UCASUL), an olive pomace oil extraction mill located in Alvito, Beja, Portugal. The provided DOR contains 8.1% moisture, 1.2% nitrogen and 0.15% phosphorus. DOR was finely powdered and kept at room temperature protected from light and moisture until further use. All results reported along this study are expressed in dry DOR basis.

2.2.1. Pre-treatment of the residues

DOR samples (50 g) were submitted to water extraction (200 mL), to isolate soluble carbohydrates, at 40 and 100 °C (DOR40 and DOR100, respectively) and with 200 mL of aqueous H_2SO_4 (1 mol dm^{-3}) at 100 °C (DOR100H), during 2 h. DOR100H was further neutralized with aqueous NaOH (1 mol dm^{-3}), and final volume adjusted to 400 mL. All of them were filtered through Whatman 100-150 filter paper under vacuum and then submitted to sterilization by autoclave at 120 °C during 22 min.

2.3. Determination of carbon sources monosaccharides concentration

Monosaccharides (glucose, xylose, arabinose, fructose and galactose) were analysed in a Hitachi HPLC equipped with a refraction index (RI) detector L-2490, a column oven L-2300, an auto-sampler L-2200, and a using a Purospher STAR NH_2 column (250 mm × 4 mm), after adequate calibration (glucose: 2.00–13.0 g L^{-1}, xylose: 0.050–2.00 g L^{-1}, arabinose: 0.050–2.00 g L^{-1}, fructose: 0.050–2.00 g L^{-1} and galactose 0.050–2.00 g L^{-1}. The eluent, aqueous acetonitrile solution 1:3,

was pumped at a flow rate of 1.0 ml min^{-1} (25 °C). The injected volume was 20 μL. All DOR aqueous extracts (DOR40, DOR100 and DOR100H) were centrifuged and filtered off with 0.22 μm filters before the analysis.

2.4. BC production and culture conditions

2.4.1. Pre-inocula preparation

The pre-inocula were prepared by growing the microorganisms at 30 °C during 48 h, in static condition, in HS liquid medium (20 g L^{-1} glucose, 5 g L^{-1} peptone, 5 g L^{-1} yeast extract, 2.7 g L^{-1} Na_2HPO_4, 1.15 g L^{-1} citric acid, agar 15 g L^{-1}, pH 5) before inoculation of 5 mL into 45 mL of liquid production medium in 250 mL Erlenmeyer flasks.

2.4.2. BC production using DOR aqueous extracts

The aqueous extracts, DOR40, DOR100 and DOR100H (45 mL) were tested in sterilized 250 mL Erlenmeyers with 5 mL pre-inocula. The initial pH value of the media was adjusted to 4.5 with NaOH 1 mol dm^{-3} [32] and was not controlled during flask cultivation. All the experiments were carried out in duplicate and under sterile conditions.

2.4.3. BC production using DOR aqueous extracts with nutrients supplementation

In specific experiments, the aqueous extracts were supplemented with KH_2PO_4 and $(NH_4)_2SO_4$. All the experiments were carried out in duplicate and the preparation of the pre-inocula and operational conditions were the same as described above.

2.4.4. BC purification and quantification

After the incubation period, the BC membranes were withdrawn from the culture media and treated with NaOH 0.5 mol dm^{-3} at 90 °C for 30 min. This procedure was repeated three times in order to eliminate attached cells [33]. Then, the membranes were washed with distilled water to remove components of the culture media and other residues until its whitening and reaching pH 7.0. Finally, the purified BC membranes were dried at 105 °C to constant weight and the concentration was determined in g L^{-1} (mass (g) of BC/volume (L) of culture medium).

2.5. Calculations

The efficiency of BC production was evaluated after 96 h of cultivation and the, substrate conversion ratio, BC production rate and BC production yield were calculated, respectively, as follows:

- Substrate conversion ratio α (%) = (Si−Sf)/Si × 100
- BC production rate r_{BC} (g L^{-1} h^{-1}) = mBC/(V.t)
- BC production yield $Y_{BC/S}$ = (mBC/V)/(Si−Sf) × 100

Where Si is the initial concentration of substrate (g L^{-1}), Sf is the final concentration (g L^{-1}), mBC is the amount of BC produced (g), V is the reaction volume (L) and t is time of cultivation (h).

2.6. Characterization of BC membranes

All bacterial cellulose membranes obtained in the different experiments were characterized in terms of surface morphology, crystallinity and structure.

Scanning Electron Microscopy (SEM) of the BC surfaces was performed using a SU-70 Hitachi instrument operating at 4 KV. Samples were previously coated with evaporated carbon.

XRD patterns of all BC membranes were measured with a Phillips X'pert MPD diffractometer using Cu Kα radiation. The crystallinity percentage was measure as χ (%) = $(I_{max}-I_{min})/I_{max}$ × 100%, where I_{max} is the height of the peak at $2\theta = 22.5$ and I_{min} the minimum between the peak at $\theta = 22.5$ and the peak at $2\theta = 16.3$.

FTIR-ATR spectra were taken with a Perkin Elmer FTIR System spectrometer equipped with a single horizontal Golden Gate ATR cell: the resolution was 4 cm^{-1} after 32 scans. Spectra were collected from 4000 to 600 cm^{-1}.

3. Results and discussion

3.1. Basic characterization of the aqueous extracts of DOR

Dry olive mill residue (DOR) was submitted to water extraction at 40 and 100 °C to prepare sugar rich aqueous extracts (DOR40 and DOR100, respectively) to be used for BC production. The total contents of monosaccharides in these aqueous extracts are shown in Table 1. Glucose, xylose and fructose were the predominant sugars detected in the both extracts. Additionally, DOR40 and DOR100, showed comparable total monosaccharides contents (11.8 and 11.9 g L^{-1} respectively) regardless of the extraction temperature used. After acid hydrolysis, and considering that the final volume of the extract (DOR100H) was doubled in relation to the previous ones due to the neutralization step, a substantial increase in the amount

Table 1 – Quantification of monosaccharides expressed in g L^{-1} of DOR aqueous extracts and in g kg^{-1} of DOR.

	Monosaccharide (g L^{-1})					Monosaccharide (g kg^{-1})				
	Xyl	Fru	Glu	Ara	Total	Xyl	Fru	Glu	Ara	Total
DOR40	0.27	1.55	10.01	–	11.83	1.08	6.2	40.04	–	47.32
DOR100	0.25	1.65	10.01	–	11.91	1	6.6	40.04	–	47.64
DOR100H[a]	3.72	0.68	4.95	1.88	11.23	29.76	5.44	39.60	15.04	89.84

a The final volume of this aqueous solution was the double of DOR40 and DOR100.

of xylose extracted, followed by arabinose, was observed, which was certainly due to the hydrolysis of xylans. In general, after hydrolysis, the total amount of monosaccharides obtained from DOR raised about 90% from 47.32 g kg^{-1} to 89.84 g kg^{-1}.

3.2. Preliminary BC production essays with DOR residues

The water extracts were then autoclaved and tested as carbon sources in BC production without the addition of any other nutrients. The results obtained (Fig. 1), showed BC productions of 0.81 ± 0.04 g L^{-1} for DOR40 and 0.85 ± 0.04 g L^{-1} for DOR100, which corresponded respectively to 32 and 34% of the production achieved with conventional HS culture medium (~2.5 g L^{-1}). Finally for DOR100H, despite the higher amounts of monosaccharides available, there was no BC production, which was certainly due to the formation or release of organic compounds such as furfural during the hydrolysis step, resulting from sugar degradation and also phenolic compounds, which could have inhibited the metabolism of *G. sacchari* and consequently BC production [3]. So, unless laborious processes for inhibitors removal are used, the hydrolysed aqueous extract DOR100H is not suited for BC production. However, the BC production values obtained for the two aqueous extracts DOR40 and DOR100 are already promising as these are considerably higher than those previously reported for other residues [22]. Based on these preliminary results it was decided that the optimization studies would be carried out with the DOR40 aqueous extract because its preparation requires less energy consumption to obtain similar results of BC production.

In order to evaluate the behaviour of bacteria in relation to the total and individual monosaccharides content of the culture media during BC production using DOR40, glucose, fructose and xylose contents were analysed.

The results obtained (Fig. 2) show that while BC produced increased overtime until 0.8 ± 0.04 g L^{-1}, the concentration in glucose decreased linearly from 10 to 2 g L^{-1}. Fructose (1.55 ± 0.08 g L^{-1}) seemed to have been nearly depleted by bacteria, during the first 30 h and the concentration of xylose remained roughly constant along the assay. The BC production presented a lag phase in the first 24 h and a linear growth until 72 h when the production entered in a deceleration phase, probably due to the lack of nutrients. However, there was still about 2 g L^{-1} glucose in the culture medium after 96 h which may suggest that content in sugars was not the limiting factor.

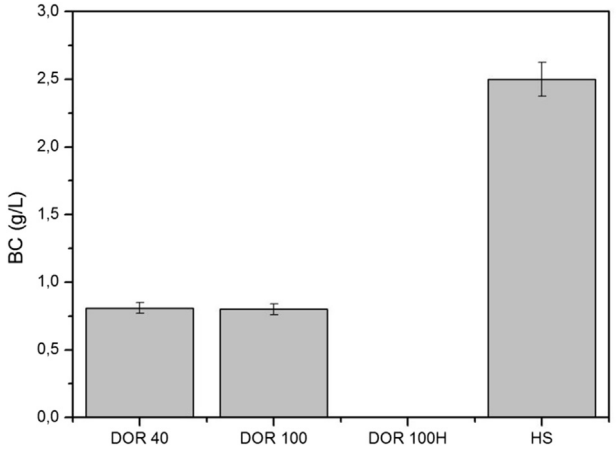

Fig. 1 – BC production using DOR40, DOR100 and DOR100H compared to the conventional HS culture medium, (error bars refer to the minimum and maximum values observed in the duplicate essays).

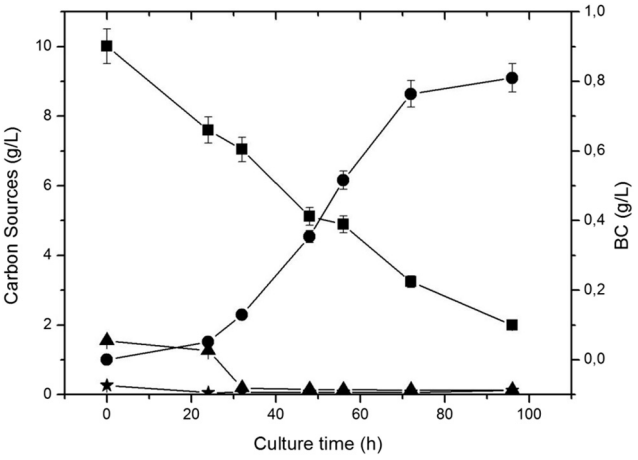

Fig. 2 – BC production (g L^{-1}) using DOR40 residue (●); glucose (■), fructose (▲) and xylose (★) (g L^{-1}) consumption overtime, (error bars refer to the minimum and maximum values observed in the duplicate essays).

3.3. BC production with DOR residues supplemented with N and P sources

In order to enhance the production of BC, the DOR40 aqueous extracts were supplemented with nitrogen (N) and phosphate (P) sources namely ammonium sulphate ($(NH_4)_2SO_4$) and potassium dihydrogen phosphate (KH_2PO_4) respectively, so that the aqueous extracts would have 1, 5 and 10 g L^{-1} of each salt respectively. The results obtained (Fig. 3), showed that the addition of a nitrogen source had a notable impact in BC production which raised more than twofold, from 0.75 ± 0.04 g L^{-1} up to 1.63 ± 0.08 g L^{-1} for the medium with 1 g L^{-1} of $(NH_4)_2SO_4$. For higher concentrations of $(NH_4)_2SO_4$, a slight decrease in BC production was observed. In relation to phosphorous supplementation, a twofold increase in BC production, after the addition of 1 g L^{-1} of KH_2PO_4 (Fig. 3) and a decrease in BC production for higher values of this nutrient were also observed. The decrease on the amount of BC produced when the concentration of both nutrients increased could be explained by the fact that other processes, namely cell growth, are also occurring in *G. sacchari* besides BC production. According to previous studies [34] the two processes occur simultaneously competing for the carbon source in *Gluconacetobacter xylinus*. By increasing the amount of nitrogen and phosphorus sources cell growth was favoured over BC

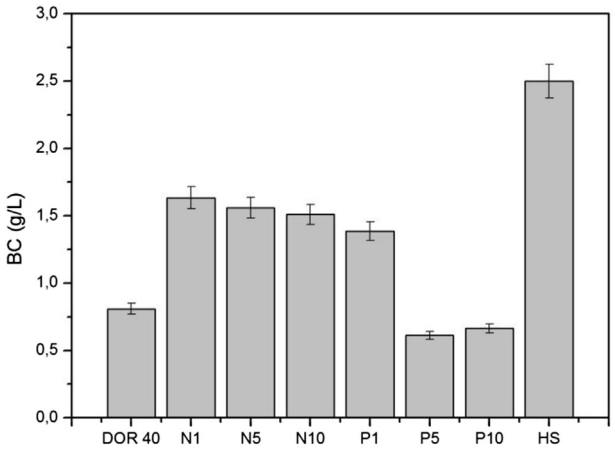

Fig. 3 – BC production from DOR40 residue supplemented with nitrogen (N) and phosphate (P), in 1, 5 and 10 g L^{-1} of each source independently, (error bars refer to the minimum and maximum values observed in the duplicate essays).

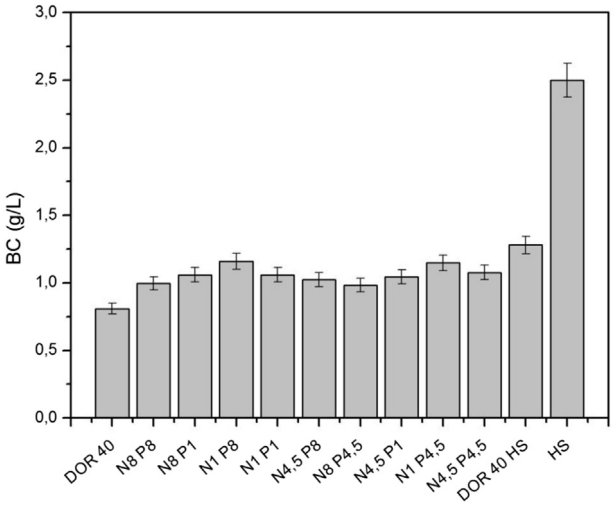

Fig. 4 – BC production from DOR40 residue supplemented with combinations of nitrogen (N) and phosphate (P), in 1, 4.5 and 8 g L^{-1} of each N and P sources independently. The BC yield of DOR40, DOR40 supplemented with HS components (except glucose source) and HS culture medium are also shown, (error bars refer to the minimum and maximum values observed in the duplicate essays).

production, resulting on the decrease of the amount of BC produced decrease for higher concentrations of $(NH_4)_2SO_4$ and KH_2PO_4. It is generally known that oxygen is critical in BC production process [35] but these results show that nitrogen and phosphorus sources could also play important roles and should be taken into account for a subsequent process optimization. However, more studies on the influence of these two nutrients and a reliable method for immediate measurement of *Gluconacetobacter saccahri* growth are required and should be developed before attempting process optimization [34,35].

In the next step, the combined effect of supplementation with N and P was accessed. As shown on Fig. 4, supplementing the DOR40 extract with N and P sources led to an increase on the BC production between 21.48% (N8 P4, 5) and 43.21% (N1 P8) when compared with no supplementation. However, these production increment results were far below from those obtained with separate N or P supplementation.

Moreover, the addition of the HS medium components, except glucose as the sugar source, to the DOR40 extract revealed a 58% increase in BC production, up to 1.28 ± 0.06 g L^{-1}. These results further confirmed the importance of such components on BC production [3]. However, this increase was not such higher than those obtained with the essays supplemented with N and P sources, to justify the use of HS components instead of individual N and P supplementations.

Finally, in order to better attempt the value of this industrial residue, the substrate conversion rates, BC production rates and yields for the extract DOR40 (with no supplementation) and of the HS medium were determined (Table 2).

The degree of conversion of substrate obtained was 82% and 90% for DOR40 and HS, respectively. In relation to the BC yield, the value was a slightly higher for the test with HS medium, 0.138 g BC per g of substrate, than for the test with DOR40 extract, 0.099 g BC per g of substrate, which represents 72% of BC production using this extract. This difference probably resulted from the fact that in HS medium, glucose was the only substrate while in DOR40 extract other sugars were present and probably yielded lower BC productions.

The productivity values obtained with DOR40, 0.008 g L^{-1} h^{-1} were in the same order of magnitude of those we have previously reported [22] using the same microorganism, but using pulp and wine residues, between (0.0013 and 0.005 g $L^{-1}h^{-1}$).

In this context [22], we have obtained a degree of conversion of substrates between 5 and 30%, which were much lower than those presented by olive oil residues, 82% and 90%.

3.4. *Characterization of BC membranes*

All BC membranes produced using DOR residue were characterized in terms of chemical structure, crystallinity and morphology by FTIR, XRD and SEM (Fig. 5), respectively. BC membranes, presented the typical FTIR spectrum of cellulosic substrates [3] with strong bands at around 3300, 2880 and 1100 cm^{-1}, associated to the vibrations of the OH, CH and C–O–C groups of cellulose. All BC membranes presented the X-ray diffraction profile of cellulose I, with the main diffraction peaks at around $2\theta = 14.9$, 16.3, 22.5, and 34.6, normally assigned to the diffraction planes 101, 10-1, 002, and 040, respectively. The crystallinity degree of these samples was around 80%. All these results were in tune with published X-ray data for BC produced by other bacteria [36].

Finally, all the studied BC samples presented the typical homogeneous tridimensional network of nano and microfibrils of cellulose as evidenced by the corresponding SEM image (Fig. 5), similar to those commonly reported for BC [4].

Table 2 – BC production rates and yields for the extract DOR40 with no supplementation and HS medium. (Si) Initial concentration of substrate, g L^{-1}, (ΔBC) amount of BC produced, (ΔS) amount of substrate consumed, g L^{-1}, (α) substrate conversion ratio, %, ($Y_{BC/S}$) BC production yield, gBC/gS, (Γ_{BC}) BC production rate or productivity, gBCL^{-1} h^{-1}.

Samples	Si (g L^{-1})	ΔBC (g L^{-1})	ΔS (g L^{-1})	α (%)	$Y_{BC/S}$ (g BC/g S)	r_{BC} (gBCL^{-1} h^{-1})
HS	20.00	2.5	18.00	90	0.138	0.026
DOR40	10.01	0.81	8.21	82	0.099	0.008

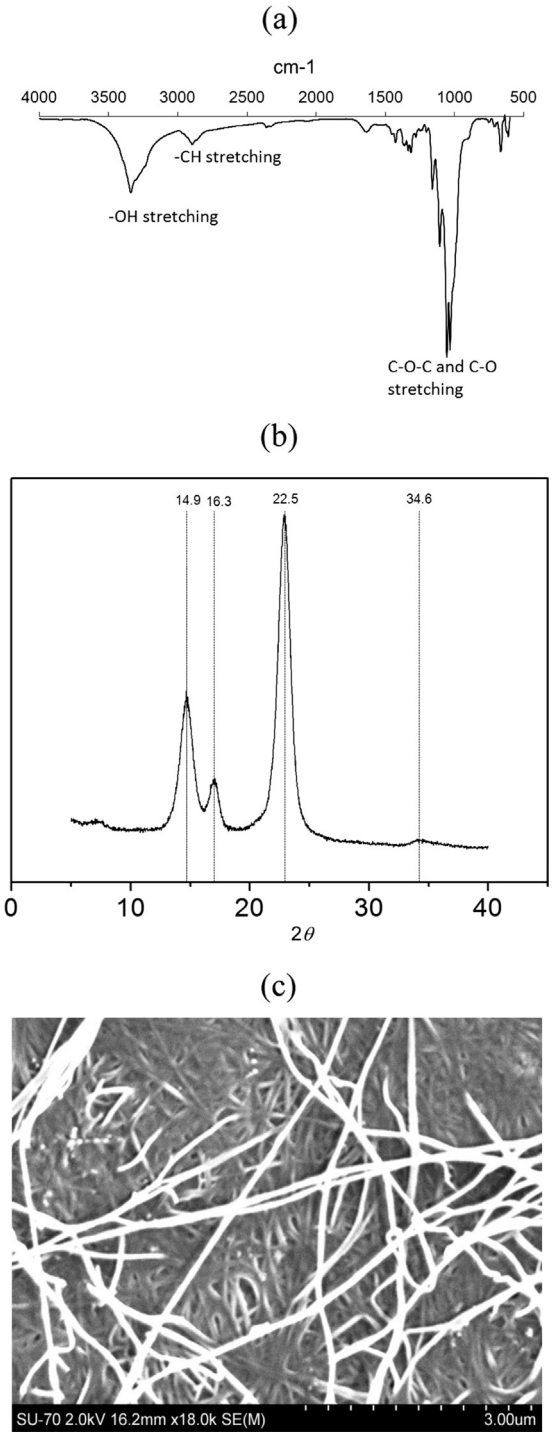

Fig. 5 – FTIR spectrum (a), X-Ray diffractogram (b) and SEM image of the BC membrane obtained with DOR40.

4. Conclusion

The present study demonstrates that dry olive mill residue (DOR) water extracts can be used as nutrients and carbon sources for the production of bacterial cellulose. Although BC was obtained in lower yields than those reported with the reference medium HS, the results are promising since we are dealing with low value residues without any kind of nutritional supplementation. Furthermore the BC production yields reported here are, so far, the highest in a process using industrial residues and *G. sacchari* bacterium. So, this work opens good perspectives for future valorization studies of these residues by producing a biomaterial with unique properties such as bacterial cellulose.

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